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# THERMOSTABLE AND THE SO-CALLED THERMOLABILE HEMOLYSINS

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F. H. Thiele and Dennis Embleton<sup>1</sup> in a paper on the "Evolution of the Antibody" present many experiments to show that the so-called amboceptor is a development of the complement, the evolution being gradually traceable from the one to the other. They used both normal and acquired hemolytic antibodies, and noticed with both that serums which were markedly hemolytic in the fresh state, not only lost this property on inactivation, but that this power was not restored or only incompletely by the addition of complement.

In their experiments with normal hemolysins advantage was taken of the natural antibodies present in 5 rabbit and 2 human serums for sheep cells. Five of these serums could not be reactivated after heating at 55 C. for 30 minutes; the other two were reactivated to a certain extent. My experiments closely corroborate these results. In 6 of their fresh serums they were able to separate the amboceptor by absorption in the cold, but report one serum in which the amboceptor and complement were both absorbed together. In my experiments summarized in this article it will be evident that in no case was a real thermolabile hemolysin found, and that with every experiment with normally hemolytic serum, amboceptor could be absorbed from the fresh serum and also from the heated, nonactivatable serum. By "thermolabile" is meant destructibility when heated in a water-bath at 56 C. for 30 minutes.

## TECHNIC

Hemolytic test tubes of 4 c c capacity were used. The required amounts of hemolytic serum, erythrocyte suspension, and complement when used, were placed in the test tubes and enough isotonic NaCl solution added to make 1 c c. After shaking, the tubes were placed in a 37 C. water-bath for 30 minutes, shaken again, and reincubated for 30 minutes. The results were then read and the tubes placed in the cold over night and any change from previous reading was noted. In the absorption experiments the tubes were cooled to between -10 C. and 0 C. in a CaCl<sub>2</sub> powdered ice mixture; the tubes when centrifuged

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<sup>1</sup> *Ztschr. f. Immunitätsf., O.*, 1914, 20, p. 1.

were kept surrounded by a sufficient quantity of the cooling mixture. The exposed erythrocytes were washed with isotonic NaCl solution cooled to 0 C.

Inactivated serum was obtained by heating the fresh serum at 56 C. for 30 minutes in a water-bath. Complement was obtained from rabbits, guinea-pigs, swine, calves, man, and dogs, and titrated before using.

The erythrocytes were washed 3 times in isotonic NaCl solution and used in dose of 0.1 cc of a 10% suspension.

In all cases complement was tested for natural hemolysin, and when amboceptor was present, as in the dog serum, it was removed by absorption with the homologous erythrocyte. The salt solution was always tested for isotonicity.

The results are expressed the same as in the Wassermann reaction. A negative (—) sign means complete hemolysis; 1 + means nearly complete hemolysis; 2 + means 50% hemolysis; 3 + means slight hemolysis; 4 + means no hemolysis.

TABLE 1  
ACTION OF DOG SERUM ON VARIOUS ERYTHROCYTES

| Serum of Dog 1                                                         | Erythrocytes |        |     |      |      |       |     |
|------------------------------------------------------------------------|--------------|--------|-----|------|------|-------|-----|
|                                                                        | Rabbit       | G. Pig | Dog | Duck | Calf | Swine | Man |
| 1. Fresh normal dog serum, 0.1 cc.....                                 | ±            | —      | 4+  | —    | —    | —     | 1+  |
| 2. Inactivated dog serum, 0.3 cc, + rabbit complement, 1 unit.....     | 4+           | 4+     | 4+  | 4+   | 3+   | —     | 4+  |
| 3. Inactivated dog serum, 0.3 cc, + swine complement, 1 unit.....      | 4+           | 4+     | 4+  | 4+   | 3+   | —     | 4+  |
| 4. Inactivated dog serum, 0.3 cc, + calf complement, 1 unit.....       | 4+           | 4+     | 4+  | 4+   | 4+   | —     | 4+  |
| 5. Inactivated dog serum, 0.3 cc, + guinea-pig complement, 1 unit..... | 4+           | 4+     | 4+  | 4+   | 4+   | —     | 4+  |
| 6. Inactivated dog serum, 0.3 cc, + human complement, 1 unit.....      | 4+           | 2+     | 4+  | 4+   | 4+   | 3+    | 4+  |
| 7. Inactivated dog serum after saturation, + complement, 1 unit.....   | 4+           | 4+     | 4+  | 4+   | 4+   | 4+    | 4+  |
| 8. Exposed erythrocytes, + complement, 1 unit .....                    | —            | —      | 4+  | —    | —    | —     | —   |

It will be observed from the results of Exper. 1 that Dog 1 had natural hemolysins for rabbit, guinea-pig, duck, calf, swine and human erythrocytes. By referring to Thiele and Embleton's work it will be seen that they absorbed the hemolysin from their fresh serum, but never attempted to absorb the hemolysins from their inactivated serum, evidently just taking it for granted that the amboceptor was destroyed from the fact that the power was not restored on the addition of fresh complement. The results shown on Lines 7 and 8 of Table 1 are of interest, as they demonstrate clearly that the hemolysin of Dog 1 serum when heated to 56 C. for 30 minutes is not affected deleteriously in the least; Line 7 shows that the inactivated serum loses all hemolysins on saturation with the respective erythrocytes; Line 8 shows that the erythrocytes (exposed) from Line 7 after saturation in the respective inactivated serum are completely laked on addition of proper complement. In this case complement was obtained by absorbing all hemol-

ysins from the fresh Dog 1 serum by excess of the respective erythrocytes and using the supernatant fluid.

The results of this experiment would seem to be in direct opposition to the argument of Thiele and Embleton for the evolution of the thermolabile complement through the so-called thermolabile antibodies to the thermostable antibodies, as it has shown that the hemolytic antibodies studied in this paper are intrinsically thermostable.

TABLE 2  
ACTION OF CALF SERUM ON VARIOUS ERYTHROCYTES

| Serum of Calf                                                                          | Erythrocytes |        |      |       |       |
|----------------------------------------------------------------------------------------|--------------|--------|------|-------|-------|
|                                                                                        | Rabbit       | G. Pig | Duck | Swine | Human |
| Fresh normal calf serum, 0.1 cc.....                                                   | —            | —      | ±    | ++    | —     |
| Inactivated calf serum, 0.2 cc, + complement, 1 unit...                                | 4+           | 4+     | 4+   | 4+    | 4+    |
| Exposed erythrocytes, after saturation in inactivated serum, + complement, 1 unit..... | —            | —      | —    | 4+    | —     |

Exper. 2 with calf blood serum merely substantiates the results of Exper. 1, namely, that the so-called thermolabile natural hemolysins are really thermostable and can be absorbed in their original power from the inactivated serum.

TABLE 3  
ACTION OF RABBIT SERUM ON VARIOUS ERYTHROCYTES

| Serum of Rabbit                                                                       | Erythrocytes |        |      |       |       |
|---------------------------------------------------------------------------------------|--------------|--------|------|-------|-------|
|                                                                                       | Rabbit       | G. Pig | Duck | Swine | Human |
| Fresh normal rabbit serum, 0.1 cc.....                                                | 1+           | —      | ±    | 4+    | 4+    |
| Inactivated rabbit serum, 0.2 cc, + complement, 1 unit.                               | 4+           | 4+     | 4+   | 4+    | 4+    |
| Exposed erythrocytes, after saturation in inactivated rabbit serum, + complement..... | —            | —      | —    | 4+    | 4+    |

The results of Exper. 3 on rabbit serum are similar to those of Exper. 1 on Dog 1 serum and to Exper. 2 on Calf serum. Kolmer and Williams<sup>2</sup> sum up very thoroughly the obvious natural hemolysins in inactivated normal rabbit serum for goat, sheep, dog, human, hog, ox, chicken, guinea-pig and white rat erythrocytes; however, they fail to take into account those hemolysins which appear to become destroyed during the heating, and it would be very interesting to know what percentage of their inactivated serums, in which they report no hemolysins, would show hemolysins on saturation in the cold with erythrocytes. As all the hemolysins studied in this article are thermostable,

<sup>2</sup> Jour. Infect. Dis., 1913, 13, p. 96.

it would be plainly a misnomer to classify part of them as thermolabile, and it is suggested, first, that all those hemolysins that can be titrated direct, including those which are still detectable after inactivation as well as those in fresh serums, be designated as "obvious" hemolysins; second, that those hemolysins that are not detectable by direct titration after inactivation be called "masked" hemolysins. These new terms will be used henceforth in this and following papers, for the sake of brevity and clearness.

I have in preparation a paper on the effect of inanition and special diets on hemolysins and an attempt will be made therein to throw some light on this curious phenomena of the obvious hemolysins being converted to the masked hemolysins by heating at 56 C. for 30 minutes, the hemolytic power remaining undiminished.

TABLE 4  
ACTION OF SWINE SERUM ON VARIOUS ERYTHROCYTES

| Serum of Swine                                                                  | Erythrocytes |        |     |      |      |       |       |
|---------------------------------------------------------------------------------|--------------|--------|-----|------|------|-------|-------|
|                                                                                 | Rabbit       | G. Pig | Dog | Duck | Calf | Swine | Human |
| Fresh normal swine serum, 0.15 cc.....                                          | 4+           | —      | 4+  | —    | 4+   | 4+    | 4+    |
| Inactivated serum, 0.3 cc, + complement .....                                   | 4+           | 4+     | 4+  | 4+   | 4+   | 4+    | 4+    |
| Exposed erythrocytes, after saturation in inactivated serum, + complement ..... | 4+           | —      | 4+  | —    | 4+   | 4+    | 4+    |

Exper. 4 with swine blood serum gives similar results to those of the three previous experiments. In all cases it will be seen that the masked hemolysins are evidently as strong as the obvious hemolysins.

TABLE 5  
ACTION OF SHEEP SERUM ON VARIOUS ERYTHROCYTES

| Serum of Sheep                                                                         | Erythrocytes |        |        |       |
|----------------------------------------------------------------------------------------|--------------|--------|--------|-------|
|                                                                                        | Dog          | G. Pig | Rabbit | Human |
| Fresh normal sheep serum, 0.1 cc.....                                                  | 4+           | —      | —      | —     |
| Fresh normal sheep serum, 0.1 cc, + guinea-pig complement, 1 unit .....                | —            | —      | —      | —     |
| Inactivated sheep serum, 0.3 cc, + complement, 1 unit.....                             | 4+           | —      | 4+     | 4+    |
| Exposed erythrocytes, after saturation in inactivated serum, + complement, 1 unit..... | —            | —      | —      | —     |

The results of Exper. 5 resemble those of the preceding four experiments. In Line 2, however, it will be noticed that the fresh sheep serum is complemented with guinea-pig serum for dog erythrocytes, but is not complemented with its own (sheep) serum. It will

be well to state here that the results of the addition of complement to a fresh serum are given only when these results are different from those when the fresh serum is used alone. H. Windsor Wade<sup>3</sup> gives two tables of statistical data concerning the relative efficiency of the Hecht-Weinberg (antisheep) and the Tschernogubow (antiguinea-pig) modifications of the Wassermann reaction; he reports on 1,000 cases (consecutive specimens obtained in routine hospital practice) and finds that of the 500 examined with the antiguinea-pig cell system only 21, or 4.2%, did not give one unit of hemolysis, and of the 500 examined with the antisheep cell system 77, or 15.4%, did not give one unit of hemolysis. In this work of Wade's no foreign complement was used; it would be very interesting to know how many of his negative cases would have caused lysis on the addition of foreign complement. Fresh serum was used in all of his experiments, hence his results show the obvious hemolysins present, but none of the masked hemolysins.

TABLE 6  
ACTION OF HUMAN SERUM ON VARIOUS ERYTHROCYTES

| Human Serum                                                                               | Erythrocytes |        |        |       |
|-------------------------------------------------------------------------------------------|--------------|--------|--------|-------|
|                                                                                           | Dog          | G. Pig | Rabbit | Sheep |
| Fresh normal human serum, 0.1 cc.....                                                     | —            | —      | —      | —     |
| Inactivated human serum, 0.2 cc, + complement, 1 unit.....                                | —            | 4+     | 4+     | 2+    |
| Exposed erythrocytes, after saturation in inactivated serum,<br>+ complement, 1 unit..... | —            | —      | —      | —     |

Kolmer and Casselman<sup>4</sup> report the results of a large number of experiments with the natural hemolysins of inactivated human serum on sheep, dog, ox, goat, hog, rat, chicken, horse, rabbit and guinea-pig erythrocytes. They found that 93% of the inactivated human serums contained natural hemolysins for sheep erythrocytes; 82% for dog; 50% for ox; 24% for goat; 12% for rat; 8% for chicken; 4% for horse and rabbit, and only 2% for guinea-pig. They concluded that the swine, rat, chicken, horse, rabbit or guinea-pig systems were preferable to the sheep or dog systems for complement fixation with human serum. The results in Line 2 of my Exper. 6 on human serum check very well with the work of Kolmer and Casselman; Line 3 shows that the hemolytic power is not destroyed by the inactivation, but has simply become masked. Note that Wade finds that 95.8% of fresh human

<sup>3</sup> Jour. Med. Research, 1916, 34, p. 113.

<sup>4</sup> Jour. Infect. Dis., 1915, 16, p. 441.

serum contain hemolysins for guinea-pig erythrocytes while Kolmer and Casselman show that only 2% of inactivated human serums contain the guinea-pig hemolysin; the difference between these percentages, or 93.8%, represents the amount of obvious hemolysins which become masked on inactivating.

Thiele and Embleton, in addition to their work with natural hemolysins, injected seven rabbits with either human or sheep erythrocytes. They found that during the first 2 or 5 days the antibody formed was inactivated by heating; then later a part of it became thermostabile and finally most of it withstood heating. They considered these results as further evidence of the "evolution of the antibody" from complement to amboceptor. To check these results I inoculated 13 rabbits with human erythrocytes; after 3 days these animals were bled and their serums tested. In 3 of these serums no hemolysins could be demonstrated; in 5 others obvious hemolysins were found, but all became masked on inactivation, which latter, however, were absorbed in the same strength as found previous to inactivation; in one other serum only part of the obvious hemolysins became masked on inactivation and the titer of the combined obvious and masked hemolysins of the inactivated serum was the same as the obvious hemolysins of the fresh serum; in the remaining 4 serums the obvious hemolysins all became masked on inactivation, but in two of these the masked titrated somewhat higher than the obvious hemolysins of the fresh serums, while in the other two the titers of the masked were slightly lower than those of the obvious hemolysins. The results of this experiment on 13 rabbits agree essentially with Thiele and Embleton's results; but my experiments show that the hemolysin is not destroyed by inactivation and demonstrate clearly that the amboceptor can be absorbed in its original power, or even stronger, from the inactivated serum. Hence, their conclusions seem to be based on a fallacy and fail to coincide with the results published here.

#### CONCLUSIONS

As all of the hemolysins investigated are thermostabile it is a misnomer to term part of them thermolabile.

In view of the foregoing fact it is suggested that all of those hemolysins which can be titrated direct be called "obvious" hemolysins.

It is also suggested further that the term "masked" be applied to those "obvious" hemolysins which are not detectable by direct titration after inactivation.

As there are no thermolabile hemolysins, there can be no "evolution of the antibody" from a thermolabile complement through a thermolabile hemolysin to a thermostable hemolysin.

The immune hemolysin first formed on injection of the antigen is thermostable.